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Increased *in situ* hybridization sensitivity using non-radioactive probes after staining for β-galactosidase activity

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Assessing gene expression patterns by using *in situ* hybridizations has become routine practise, both in wild-type and mutant model organisms. With the increased use of mouse knock-out and transgenic lines carrying the *nlacZ* (n=nuclear localization signal) reporter gene, combining *in situ* hybridization and β-galactosidase activity is often required to compare accurately expression patterns in order to investigate gene function (Ref. 1, 2). However, a significant loss in sensitivity for transcript detection after staining has hampered studies in the field. Some of these problems could be circumvented by using *in situ* hybridizations with ^{35}S-labelled nucleotides (Ref. 1). However, radioactive *in situ* hybridizations can only be performed on sections, and single-cell resolution is facilitated using non-radioactive *in situ* hybridizations. Here, we describe a protocol that significantly improves transcript detection following detection of β-galactosidase activity, both on whole mount and on sectioned samples. First, we reasoned that staining for β-galactosidase activity provoked transcript loss from the tissue in spite of its fixation prior to staining. Given that fixation preserves tissue integrity and stabilizes transcripts, we included fixative in the β-galactosidase-staining solution. This appeared to be crucial for increased *in situ* hybridization sensitivity. Since fixative destroys β-galactosidase enzymatic activity, fixation times prior to staining and the concentration of fixative employed during staining should be dosed for weakly expressing *lacZ* lines. Second, the β-galactosidase substrate is dissolved in dimethylsulfoxide (DMSO), which might be responsible for this transcript loss; therefore, the substrate was dissolved at a higher concentration, effectively reducing the final DMSO concentration. Finally, including Tween-20 in the fixative prior to staining for β-galactosidase also increased the sensitivity, presumably by rendering the tissue more accessible to the fixative. The protocol is outlined below. All solutions and instruments were sterile to preserve RNA integrity.

For a number of *nlacZ* lines that we have investigated, the protocol described was sufficient to observe a dramatic improvement, in particular for sections where previously, little to no transcript was detected using non-radioactive probes. On whole mount, including the modifications below increased the sensitivity of the signal approximately four to seven fold. In some cases this made the difference between observing a signal and not. When compared to embryos not stained for β-galactosidase, the sensitivity of the signal using the protocol described was lowered by approximately two fold. On sections, for most probes, we did not obtain an *in situ* signal if the modifications indicated were not included. For single-cell resolution, the discrimination of the respective signals depends on the relative size of the cytoplasm to the nucleus. The use of a *lacZ* with a nuclear localization signal might also be useful, although some diffusion of this signal occurs after the *in situ* hybridization.

Methods

1. Mouse embryos were isolated in sterile PBS/2 mM EGTA and fixed as rapidly as possible in fresh 4% paraformaldehyde (PFA) or formaldehyde (containing

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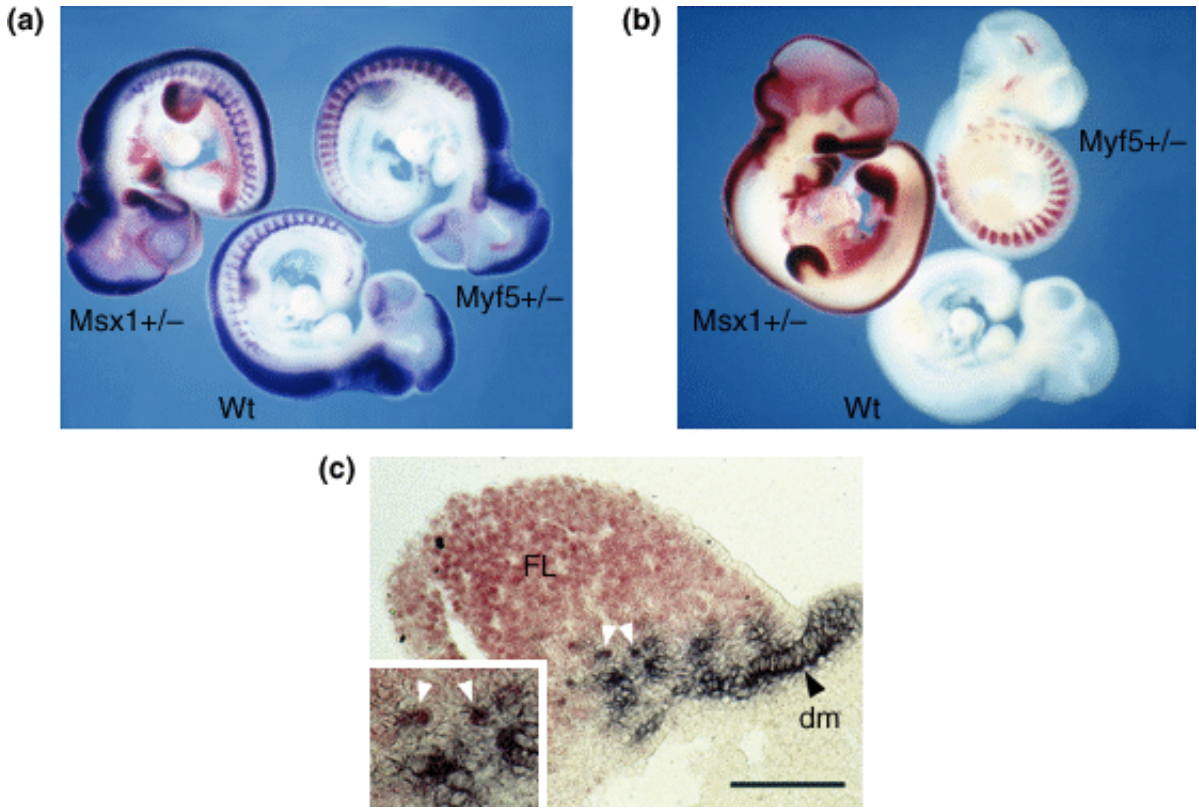

FIGURE 1. Embryos (embryonic day E10.5) that were wild type (wt) or contained an *nlacZ* knock-in into the *Myf5* (Ref. 2) or *Msx1* (Ref. 5) loci were prepared as indicated, either with (a; also including Tween-20 during the initial fixation) or without (b) fixative in the β-galactosidase-staining solution (Salmon-gal, pink). Embryos (a and b) were then hybridized with a *Pax3* antisense probe. Note the Pax3 signal (purple) in the neural tube and somites of the embryos in (a) but not in (b). The experiments shown in (a) and (b) were performed in parallel, under identical conditions and the reactions were stopped at the same time. The staining reaction for Pax3 transcripts was stopped prior to completion (9 h) in order to highlight the differences. (c) E9.5 *Msx1* heterozygote embryo was cryostat sectioned (Ref. 2), stained with Salmon-gal and hybridized with a Pax3 antisense probe. The inset is an enlargement indicating colocalization of $Msx1^+$ (pink, arrowheads) and $Pax3^+$ (purple) cells. FL, forelimb; dm, dermomyotome.

0.1% Tween-20 for whole embryos) at 4°C with gentle agitation. Fixation times were about 45–60 min for E9.5 and 60 min for E10.5 to E11.5 embryos.

2. Embryos were rinsed in PBS for 10 min then stained for β-galactosidase activity at 37°C until the desired intensity was reached (several hours) as follows:

   10 ml PBS containing:

   200 μl of 200 mM potassium ferricyanide (aliquots stored at −20°C; 2 mM final)

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   40 μl of 100 mg/ml X-gal (blue-green precipitate; Gibco, BRL) or Salmon-gal (pink-red precipitate; Apollo Scientific Ltd) in DMSO (400 μg/ml final; aliquots stored at −20°C). Salmon-gal is recommended owing to the better contrast with the alkaline phosphate purple substrate. However, penetration of whole embryos older than E11 is not complete. Add these substrates before adding $MgCl_2$ to facilitate dissolution, and vortex (if not, dissolve stock at 80 mg/ml)

   20 μl of 1 M magnesium chloride (2 mM final concentration)

   100 μl 10% Tween-20 (0.1% final concentration)

   500 μl 4% fresh PFA (250 μl for sections; final concentrations 0.2% and 0.1%, respectively)

3. Embryos were rinsed in PBS twice for 5–10 min then refixed in 4% PFA at 4°C with gentle agitation.

4. Embryos were rinsed in PBT (PBS/0.1% Tween-20) for 5 min at room temperature, dehydrated in 50% methanol in PBT (5 min), and twice in 100% methanol, then stored at −20°C for several months. Long storage, however, compromises sensitivity.

5. *In situ* hybridization was carried out on whole embryos as described (Ref. 3), with modifications (Ref. 2) or on sections (Ref. 4). Figure 1 shows some typical results.

Acknowledgements

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Products Used

X-gal: X-gal from Boehringer Mannheim
X-gal: X-gal from Life Technologies (Gibco BRL)
blue-green precipitate: blue-green precipitate from Life Technologies (Gibco BRL)